Marine Biology (1997) 130: 141–149

M. B. Black · K. M. Halanych · P. A. Y. Maas
W. R. Hoeh · J. Hashimoto · D. Desbruyères
R. A. Lutz · R. C. Vrijenhoek

Molecular systematics of vestimentiferan tubeworms from hydrothermal vents and cold-water seeps

Received: 11 July 1997 / Accepted: 21 August 1997

Abstract Vestimentiferan tubeworms inhabit sulfide-rich environments associated with deep-sea hydrothermal vents and cold-water seeps at continental margins. Twelve species have been described, and several more await formal descriptions. As a group, these worms are best known for their lack of a digestive system in adults and their dependence on endosymbiotic bacteria that supply nutrients derived from chemoautotrophism. The taxonomic status of Vestimentifera has been debated since their discovery. Furthermore, relationships within the Vestimentifera have been difficult to determine by morphological criteria. Several species display considerable phenotypic plasticity, further confounding efforts to establish evolutionary relationships. We used a fragment of the mitochondrial cytochrome c oxidase subunit I gene to examine evolutionary relationships among vent-endemic species (*Riftia pachyptila*, *Oasisia alvinae*, *Ridgeia piscesae*, *Tevnia jerichonana*) and seep-associated species (*Escarpia laminata*, *E. spicata*, *Lamellibrachia barhami*, *L. columna*, and an undescribed species) of these worms. The molecular data placed these vestimentiferan taxa within the phylum Pogonophora. The pogonophoran clade (including vestimentiferans) was then linked to the Annelida. Examination of sequence divergence suggests that extant vestimentiferans constitute a recent evolutionary radiation that diversified as a paraphyletic assemblage of seep-associated taxa (including the genera *Lamellibrachia* and *Escarpia*) and then gave rise to a clade of vent-endemic taxa (genera *Riftia*, *Oasisia*, *Ridgeia* and *Tevnia*).

Communicated by N.H. Marcus, Tallahassee

M.B. Black[1] · K.M. Halanych · P.A.Y. Maas ·
W.R. Hoeh[2] · R.A. Lutz · R.C. Vrijenhoek (✉)
Center for Theoretical and Applied Genetics
and Institute of Marine and Coastal Sciences,
Rutgers University, New Brunswick,
New Jersey 089903-0231, USA

J. Hashimoto
Japan Marine Science and Technology Center (JAMSTEC),
2-15 Natsushima-Cho, Yokosuka 237, Japan

D. Desbruyères
URM No. 7, Département Environnement Profond,
IFREMER, BP 70, F-29280 Plouzané Cedex, France

Present addresses:
[1] Maurice Lamontagne Institute, PO Box 1000,
Mont-Joli, PQ.G5H 3Z4, Canada
[2] Department of Zoology, Miami University, Oxford,
Ohio 45056, USA

Introduction

Vestimentiferan tubeworms are among the dominant fauna associated with hydrothermal vents in the Pacific Ocean. They also occur in sulfide-rich cold seeps along continental margins. As adults they have no mouth, gut, or anus, and depend on sulfur-oxidizing bacterial endosymbionts for nutriment (Cavanaugh et al. 1981; Felbeck 1981). Webb (1969) erected the order Vestimentifera to encompass these worms, which he considered as members of the class Pogonophora (Phylum: Annelida). Descriptions of new vestimentiferan species quickly followed the discovery of hydrothermal vents in the eastern Pacific. In revising the taxonomy of these unusual worms, Jones (1985) recommended the Vestimentifera be elevated to phylum level and considered a sister taxon to the phylum Pogonophora (cf. Mañé-Garzón and Montero 1985; Jones 1987).

Southward (1988, 1991, 1993) does not recognize Jones' (1985) new phylum. She treats vestimentiferans as a discrete class (the Obturata) of the phylum Pogonophora. Earlier, Jones (1981) had also recognized vestimentiferans as members of the Pogonophora. He placed vestimentiferans in a new subphylum, the Obturata, and relegated all other pogonophorans to the subphylum Perviata. Based on morphology, vestimentiferans and perviate pogonophorans have been linked to the Annelida (Nørrevang 1970, 1974; Van der Land and Nørrevang 1975; Bartolomaeus 1995). Some authors (Webb 1964; Ivanov 1988; Southward 1988; Jones and Gardiner 1989) base this relationship partly

on a free-feeding trochophore-like larval stage, but this primitive characteristic is shared by many metazoan taxa. The association with annelids remains inconclusive (Rouse and Fauchald 1995). Ivanov (1988, 1994) treats the Pogonophora (incorporating vestimentiferans) as distinct from the phylum Annelida. Crucial questions remain, however, regarding development of the coelom, the nervous and circulatory systems, and segmentation in pogonophorans and vestimentiferans (Southward 1993).

Molecular systematics offers an alternative approach to inferring evolutionary relationships among taxa that have proven difficult to resolve with morphological and developmental studies. Recent studies involving mitochondrial cytochrome oxidase I and nuclear ribosomal sequences support a close affiliation between vestimentiferans and perviate pogonophorans (Winnepenninckx et al. 1995). However, the exact placement of a vestimentiferan and pogonophoran clade among protostome metazoans is questionable. Although some studies ally these organisms with annelids (Field et al. 1988; Suzuki et al. 1988, 1989, 1993; Kojima et al. 1993), Winnepenninckx et al. suggested that pogonophorans and vestimentiferans are related to echiurans. For ease of discussion, we will assume that the vestimentiferans are within the pogonophoran clade, unless otherwise stated.

Evolutionary relationships within the Vestimentifera also are poorly resolved. Twelve species are assigned to eight genera and seven families. The lack of shared-derived characters between taxa has led to a preponderance of monogeneric families (six) and monospecific genera (six). Furthermore, some taxa exhibit extraordinary morphological plasticity that has misled several investigators to propose new species that did not withstand the scrutiny of subsequent molecular analyses. For example, two morphotypes of the genus *Ridgeia* were described as distinct species (Jones 1985), and six additional morphospecies were suspected from vents along the Juan de Fuca/Gorda/Explorer Ridge system (Tunnicliffe 1983, 1988; Tunnicliffe et al. 1985,1986). Nevertheless, an allozyme survey detected no evidence for reproductive isolation or substantive geographical subdivision among these morphotypes (Black 1991). These morphotypes are now included in a single polytypic species, *R. piscesae* (Southward et al. 1995). Morphological plasticity also occurs in the polytypic vent tubeworm *Riftia pachyptila* (Black et al. 1994). Recognition of this plasticity does not preclude, however, the existence of cryptic species in other tubeworm taxa. For example, cryptic species are common among vent- and seep-endemic vesicomyid clams (Vrijenhoek et al. 1994; Peek et al. 1997).

Questions also exist concerning the evolutionary age of vestimentiferan tubeworms, as these soft-bodied animals have left a limited and ambiguous fossil record. Some phylogenetically ancient relicts, notably stalked barnacles and slit limpets, flourish at vents, and thus have led some investigators to hypothesize that vent habitats served as refugia for ancient lineages that otherwise failed to survive mass extinctions in the photic zone (McLean 1985; Newman 1985). A well-preserved Silurian vent community with fossilized tubes was thought to contain vestimentiferans (Little et al. 1997). Fossilized "tubeworm assemblages" were reported from fossilized vent sites as old as the mid to late Carboniferous (~325 million years ago), but these tubes may have been left by a variety of marine invertebrates, ranging from polychaetes to holothurians (Tunnicliffe 1991). Less controversial reports of fossilized vestimentiferan tubeworms are from the late Cretaceous Samail ophiolites in Oman (Haymon and Sinclair 1984; Haymon and Koski 1985).

Our goals in this study were: (1) to elucidate higher-level evolutionary relationships between vestimentiferan tubeworms and other protostome taxa; (2) to characterize genetic diversity and evolutionary relationships among vestimentiferan lineages; and (3) to assess the evolutionary age of vestimentiferans as a group. To accomplish these goals, we provide new DNA sequences from vestimentiferan specimens representing eight species and six genera. Representatives of four protostome phyla were used as outgroups for the higher level phylogenetic comparisons. All analyses were based on a 650 base pair (bp) region of the mitochondrial cytochrome oxidase subunit I gene (COI).

Materials and methods

Specimens examined in this study were collected during several oceanic cruises (1992, 1994 and 1995) by one or more of the authors, and the collections and voucher specimens are stored at Rutgers University. Several of the outgroup taxa were purchased as living specimens from biological supply companies (see Table 1). Specimens were either frozen at −80 °C immediately upon collection and DNA was extracted subsequently at Rutgers University, or DNA was extracted from fresh tissue and the remaining carcasses are in −80 °C storage. The two *Ridgeia* specimens from the Southern Explorer Ridge (the ridge system immediately to the north of the Juan de Fuca Ridge and separated by the Sovanco Transform fault) were collected in July of 1994. One specimen came from a collection that falls within the known range of morphological variation for *R. piscesae* (Jones), while the other came from an adjacent collection which did not. This second morphotype is tentatively referred to as *Ridgeia* sp. until the collection can be analyzed in greater detail. *Escarpia spicata* (Jones) was collected at a cold-water seep site and a hydrothermal vent site in the Guaymas Basin in May 1995. All vestimentiferan specimens were identified morphologically by MBB prior to DNA analyses. The identification of *Galathealinum brachiosum* (Ivanov) was made by Dr. E. Southward at the time of collection, and later confirmed in her laboratory.

Molecular methods

Total genomic DNA was extracted using a modified hexadecyltrimethyl-ammonium bromide (CTAB) protocol (Doyle and Dickson 1987). A ≃710 base pair region of the mitochondrial cytochrome c oxidase subunit I gene was amplified via PCR using the primers LCO1490 and HCO2198 described in Folmer et al. (1994). The 50 μl PCR (polymerase chain-reactions) consisted of 35 to

70 ng of template DNA, 5 μl of the 10× buffer supplied by the manufacturer, 2.5 m*M* $MgCl_2$, 200 μ*M* dNTPs, 0.5 μ*M* of each primer, and 1.5 U *Taq* polymerase (Promega Inc., Wisconsin). After an initial denaturation at 95 °C for 2 min, reactions were amplified during 35 cycles as follows: 1 min at 95 °C, 1 min at 40 °C, and 1.5 min at 72 °C, followed by a final extension step of 7 min at 72 °C. For each set of PCR reactions, negative controls and sterile laboratory techniques were employed. PCR products were isolated with 1.5% NuSieve (FMC Bioproducts, New Jersey) agarose gels stained with ethidium bromide. The gel-isolated products were purified using either the Wizard-PCR kit (Promega, Wisconsin) or the Qiagen gel extraction kit (Chatsworth, California).

The sequences reported here were originally obtained with "manual" sequencing methods. However, due to the presence of some ambiguities, all sequences (except the *Riftia pachyptila* – 9°N, East Pacific Rise specimen) were verified by automated sequencing. In both cases, the LCO1490 and HCO2198 oligonucleotides served as sequencing primers.

For the manually obtained sequences, 55 ng to 80 ng purified PCR product was sequenced with the Ampli*Taq* cycle sequencing kit (Perkin-Elmer Cetus, California). The cycle-sequencing parameters were as follows: 20 cycles of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1.5 min. The primers were end-labeled with $\gamma^{33}P$ to allow visualization of fragments that were separated by a 5 to 6% denaturing polyacrylamide gel. For each primer, short- and long-sequence reads were combined and light- and heavy-strand reads compared using the sequence editor Gene Runner (Version 3.0, Hastings Software, Hastings, New York).

The automated sequence reactions followed the manufacturer's (FS Dye Termination Mix, Applied Biosystems Inc., California) recommendations using ≃60 ng of purified PCR product as template. The reaction profile was 25 repetitions of denaturation at 96 °C for 30 s annealing at 50 °C for 15 s, and extension at 64 °C for 4 min. Fragments were visualized by use of dye-labeled terminator nucleotides. The samples were electrophoretically separated on a Perkin Elmer ABI 373A DNA sequencer. Data were analyzed using the AutoAssembler and Sequence Navigator programs (Applied Biosystems Inc., California). Both strands of the PCR product were completely sequenced. A comparison of the manually obtained sequences to the automated sequences allowed for the resolution of the ambiguous sites.

Analyses

Table 1 lists the operational taxonomic units (OTUs) examined, their collection locality, the identifying label used throughout this paper, and their GenBank accession number. Final sequences were entered into the Eyeball Sequence Editor editor (ESEE; Cabot and Beckenbach 1989), and aligned using the amino acid sequences of the following reference taxa: *Drosophila yakuba* (GenBank Accession X03240; Clary and Wolstenholme 1985), *Daphnia pulex* (GenBank Accession Z15015; Van Raay and Crease 1994), *Anopheles gambiae* (GenBank Accession L20934; Beard et al. 1993) and *Artemia franciscana* (GenBank Accession X69067; Perez et al. 1994). The alignment consisted of 650 nucleotide positions, corre-

Table 1 Taxa used in analysis of a 710 base pair fragment of mitochondrial cytochrome oxidase subunit I gene (*OTU* operational taxonomic unit)

Organism	Source	OTU label	GenBank accession
Vestimentifera			
Axonobrachia			
Riftia pachyptila	9°N Eastern Pacific Rise (09°31′N, 104°18′W; 2564 m)	*Rpa* 9N	U74074
Riftia pachyptila	18°S Eastern Pacific Rise (18°36′S, 113°24′W; 2673 m)	*Rpa* 18S	U74053
Basibranchia			
Escarpia laminata	Florida Escarpment (26°02′N, 84°54′W; 3310 m)	*Ela*	U74063
Escarpia spicata	Guaymas Basin seep (27°35′N, 111°28′W; 1653 m)	*Esp* seep	U74065
Escarpia spicata	Guaymas Basin vent (27°00′N, 111°25′W; 2020 m)	*Esp* vent	U74064
Lamellibrachia barhami	Middle Valley (48°27′N, 128°42′W; 2416 m)	*Lba* MV	U74055
Lamellibrachia barhami	Oregon Margin (44°41′N, 125°17′W; 2089 m)	*Lba* OM	U74054
Lamellibrachia columna	Lau Basin (22°32′S, 176°43′W; 1870 m)	*Lco*	U74061
Oasisia alvinae	21°N Eastern Pacific Rise (20°47′N, 109°09′W; 2577 m)	*Oal*	U74069
Ridgeia piscesae	Juan de Fuca Ridge (44°39′N, 130°22′W; 2227 m)	*Rpi* SJR	U74073
Ridgeia piscesae	Southern Explorer Ridge (49°46′N, 130°16′W; 1780 m)	*Rpi* SER	U74057
Ridgeia sp.	Southern Explorer Ridge (49°46′N, 130°16′W; 1780 m)	*Rsp* SER	U74056
Tevnia jerichonana	9°N Eastern Pacific Rise (09°50′N, 104°17′W; 2550 m)	*Tje*	U74075
Undescribed species	Nikko Seamount (23°04.6′N, 142°19.8′E; 433 m)	Nikko vest.	U74078
Pogonophora			
Galatheolinum brachiosum	Oregon Margin (45°01′N, 125°17′W; 2028 m)	*Gbr*	U74066
Undescribed species	Loihi Seamount (18°55′N, 155°15′W; 915 m)	Lohi pogo.	U74068
Annelida			
Hirudinea			
Hirudo medicinalis	Purchased from Carolina Biological Supply Co.	*Hme*	U74067
Oligochaeta			
Tubifex tubifex	Purchased from Carolina Biological Supply Co.	*Ttu*	U74076
Polychaeta			
Paralvinella palmiformis	Jaun de Fuca Ridge (47°58.2′N, 129°05.2′W; 2180 m)	*Ppa*	U74070
Echiura			
Urechis sp.	Purchased from Pacific Bio Marine Supply Co.	*Urechis* sp.	U74077
Sipuncula			
Phascolosomida			
Phascolosoma sp.	Purchased from Pacific Bio Marine Supply Co.	*Phoscolosoma* sp.	U74071

sponding to 216 amino acid residues which contained only one amino acid deletion in *D. pulex*. The inferred amino acid sequences were obtained using the *Drosophila* mitochondrial translation code.

Phylogenetic analyses were performed using maximum parsimony (MP), maximum-likelihood (ML), and neighbor-joining (NJ) for both nucleotide and amino acid residues. PAUP (phylogenetic analysis using parsimony; Version 3.1.1; Swofford 1993) was used to conduct the parsimony analyses. Branch-and-bound searches were used to find the best tree(s) for the nucleotide data set, while heuristic searches were used in all parsimony bootstrap-analyses and to find the best amino acid tree. Neighbor-joining analysis was completed using the phylogeny inference (PHYLIP) package (Version 3.5c; Felsenstein 1993) and employed a Kimura two-parameter correction model. The nucleotide likelihood analyses were conducted using fastDNAml (Olsen et al. 1994) with a Kimura two-parameter correction. Protein maximum likelihood (PROTML: Version 2.2; Adachi and Hagesawa 1992) was used to obtain the likelihood tree for the amino acid data based on a Jones–Taylor–Thornton (Jones et al. 1992) amino acid substitution matrix. Bootstrap analysis for both parsimony and neighbor-joining consisted of 500 iterations, whereas the likelihood analysis only employed 100 iterations (due to computation time limitations). Computation time and software limitations made a likelihood bootstrap of the amino acid data prohibitive.

Because of the problem of third-position transitional saturation of protein coding mitochondrial genes (Brown et al. 1982; Graybeal 1993; Adkins and Honeycutt 1994; Yoder et al. 1996), the MacClade (Version 3.06; Maddison and Maddison 1992) software package was used to recode third-position nucleotides to remove transitional state changes prior to all analyses of the nucleotide data.

Results

Our aim was to obtain as complete a representation as possible of the Vestimentifera. The following taxa were not available due to their rarity: the undescribed Vigo worm (Dando et al. 1992; Williams et al. 1993), *Alaysia spiralis* (Southward 1991), *Lamellibrachia luymesi* (Van der Land and Nørrevang 1975) and *L. victori* (Mañé-Garzón and Montero 1985). While pogonophoran species have been reported from all the world oceans, they are often found in remote regions and thus are not easily collected.

Due to the uncertainty of vestimentiferan/pogonophoran relationships (Kojima et al. 1993; Winnepenninckx et al. 1995), we examined a variety of protostome taxa to determine appropriate outgroup taxa. First, we reconstructed these higher level relationships by taking advantage of the conservative nature of amino acid substitutions in the COI gene. Subsequent analyses focusing on the ingroup used the original nucleotide data, with third positions recoded to remove transitions.

Of the 216 inferred amino acid (a.a.) positions, 118 were variable and 81 were informative (with respect to parsimony analysis) across the full range of protostomes examined. However, within the Vestimentifera, we found only one parsimony-informative a.a. substitution (Position 139) which suggests that *Riftia* and *Tevnia* are sister taxa. Although different outgroup topologies were obtained with maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods, all reconstructions placed annelids as the closest pogonophoran relatives. Fig. 1a shows the maximum-likelihood reconstruction obtained from these a.a. sequences (log likelihood score = −2152.19). Based on previously published phylogenies (e.g. Eernisse et al. 1992; Halanych et al. 1995), we rooted the topology along the branch separating arthropods from the remaining protostome taxa. The results of bootstrap analyses are shown on the topology. Although we prefer ML as a method of phylogeny estimation (see Swofford et al. 1996), the NJ topology (Fig. 1b) is more consistent with the current understanding of protostome phylogeny. For example, the two crustaceans are monophyletic. Parsimony analysis (heuristic search option with tree-bisection-reconnection branch swapping) identified 148 "shortest" trees (369 steps; confidence interval, CI = 0.797, CI minus uninformative characters = 0.601). Most variation among these trees was due to the zero-length branches within the vestimentiferan clade, and the strict consensus of these trees was identical to the ML reconstruction except that the echiurid (*Urechis* sp.) and the scipunculan (*Phascolosoma* sp.) fell out as paraphyletic with the sipunculan more basal. All three methods were similar with respect to placement of the pogonophoran clade and the polychaete annelid *Paralvinella* sp. at the base of the pogonophoran clade.

Based on these amino acid reconstructions, the taxa employed in the analyses of nucleotide data were trimmed to include the Pogonophora and the three annelid taxa as outgroups (19 total taxa). The nucleotide data included 287 variable positions and 202 parsimony-informative sites when third-position transitions were excluded. These values fell to 164 variable and 111 informative sites upon exclusion of the annelid outgroups.

All three methods of phylogeny reconstruction (MP, ML, and NJ) recovered the same topology suggesting that the signal in the data is robust under a variety of assumptions. This topology (shown in Fig. 2) includes proportional branch lengths obtained by likelihood reconstruction (log likelihood score = −5861.38). The most parsimonious tree obtained with the PAUP branch and bound algorithm consisted of 585 steps, with a CI of 0.568 (CI minus uninformative characters = 0.484). The bootstrap values for all three reconstruction methods are shown in Fig. 2. In general, the tips of the tree were well supported, whereas deeper nodes within the Pogonophora had lower bootstrap support.

Distance values based on a Kimura two-parameter correction of the nucleotide data are shown in Table 2. For the purpose of comparison with published data, the reported distance values were based on the original nucleotide data (i.e. not recoded third positions). The number of absolute differences between OTUs is also shown.

Discussion

The present results with mitochondrial COI sequences indicate that vestimentiferan tubeworms are nested

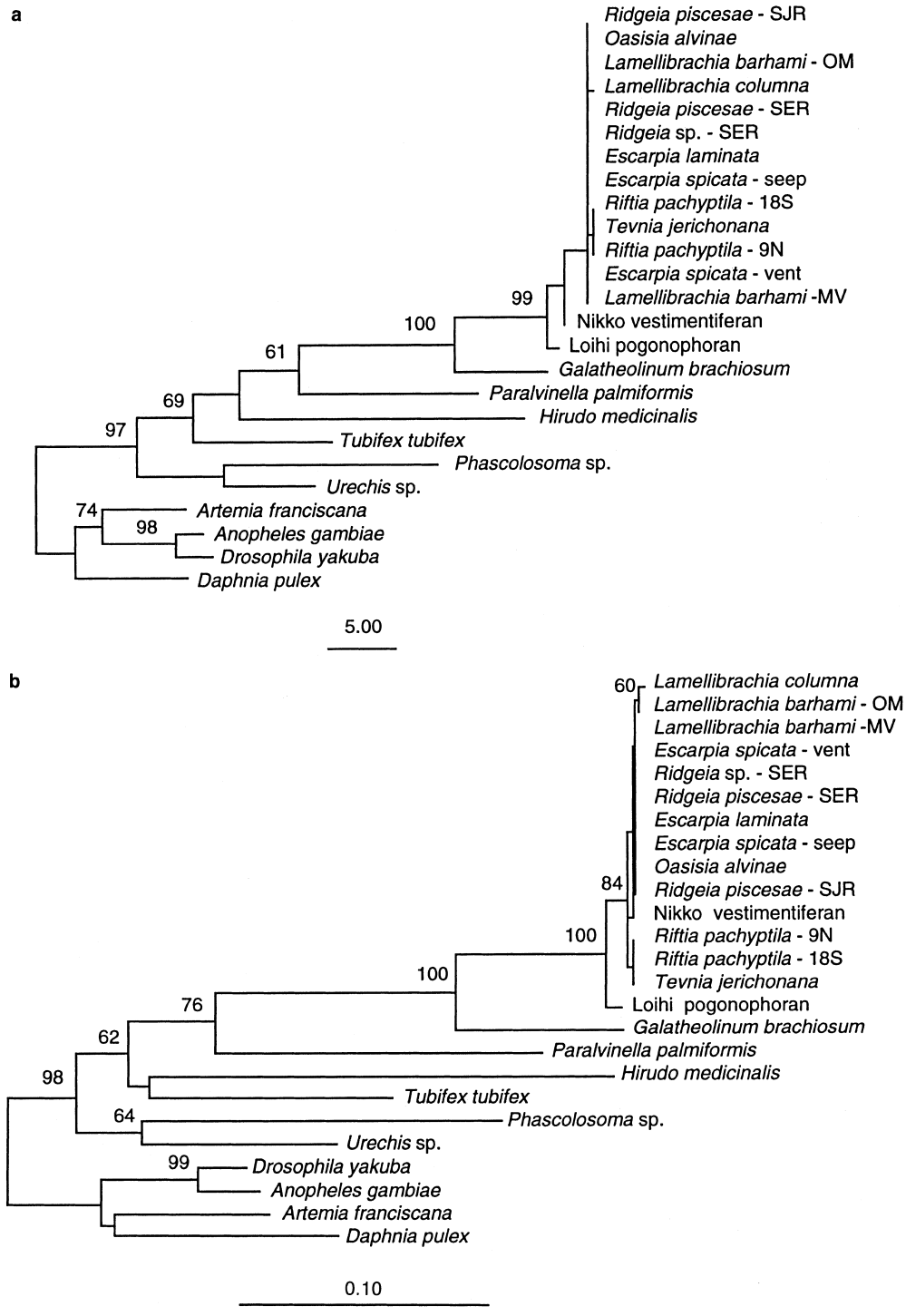

Fig. 1 Trees based on cytochrome c oxidase subunit I, inferred amino acid sequences (216 residues). **a** Protein maximum-likelihood tree (PROTML Version 2.2) with branch lengths estimated from a Jones–Taylor–Thornton substitution matrix (1992) and a STAR decomposition search; parsimony bootstrap values (500 iterations, see "Results") and a scale bar of branch length as percent probability of change along a given branch are shown; unfortunately, due to computation time and software limitations, a maximum-likelihood bootstrap analysis was not possible. **b** Neighbor-joining tree based on a PAM matrix; branch lengths represent corrected amino acid distances calculated with ProtDIST program in PHYLIP package (*scale bar* = percent change) (*Numbers* proportion of occurrence in 500 neighbor-joining replicates, bootstrap values of $<60\%$ are not shown for either **a** or **b**; *OTU labels* as in Table 1)

within the Pogonophora. Recent analysis of nuclear small-subunit (18S) rDNA sequences also identified a close relationship between perviate pogonophorans and vestimentiferans, and supported inclusion of the two taxa in a single clade (Winnepenninckx et al. 1995). However, basing their findings on the nuclear 18S rDNA, Winnepenninckx et al. suggested that pogonophorans may be more closely related to echiurans than annelids. The present phylogenetic reconstructions based on COI indicate that pogonophorans share an evolutionary history with annelids, and place echiurans outside this group (Fig. 1). In all our reconstructions, one or more of the annelid taxa (typically the polychaete *Paralvinella palmiformis*) occurred immediately basal to the Pogonophora. This placement is consistent with other phylogenetic analyses (Bartolomaeus 1995; Rouse and Fauchald 1995; Malakhov et al. 1996; McHugh 1997).

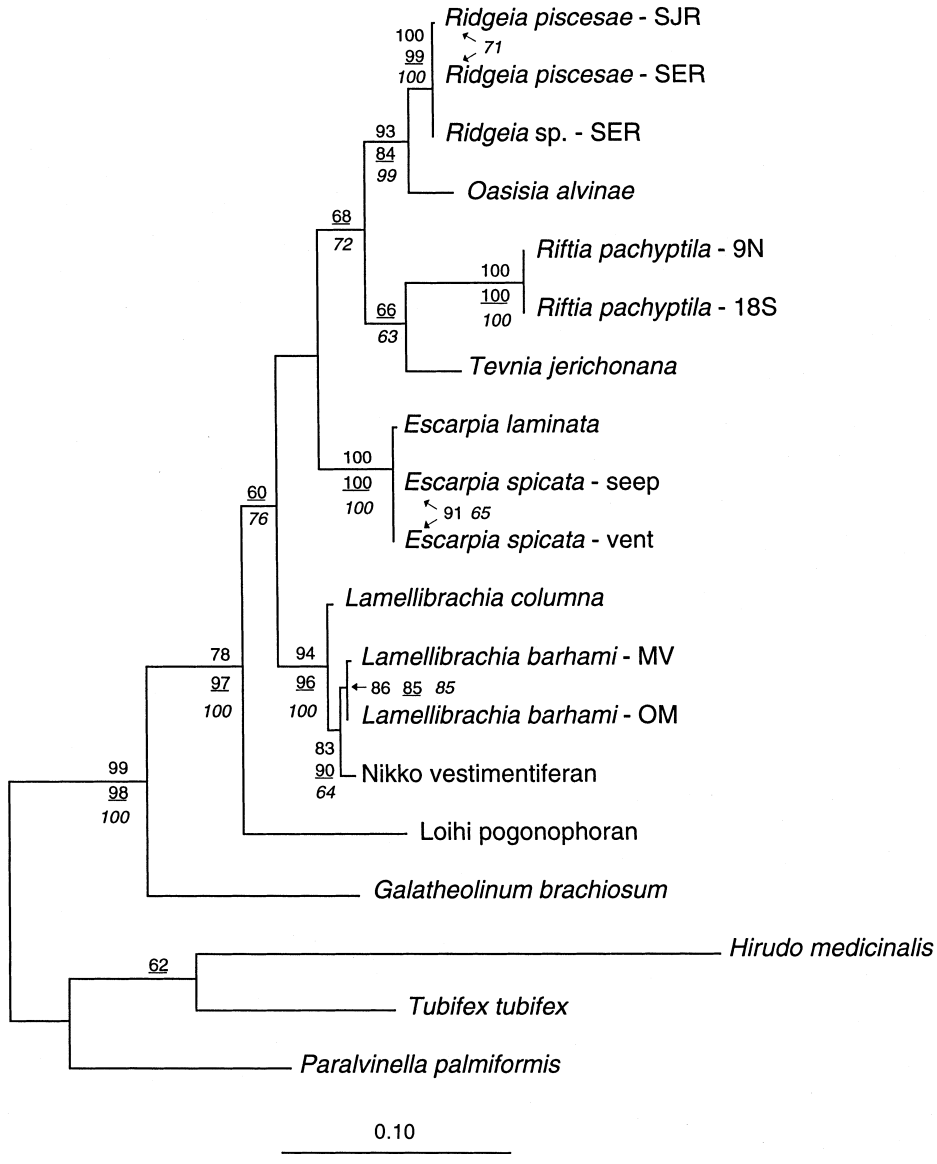

Fig. 2 Tree based on cytochrome c oxidase subunit I, DNA sequences (650 base pairs – recoded to remove third codon position transitions; *OTU labels* as in Table 1). Topology obtained with parsimony (PAUP, branch and bound search, TBR branch-swapping), maximum-likelihood (PHYLIP, Kimura two-parameter model), and neighbor-joining (PHYLIP, Kimura two-parameter model) reconstruction algorithms [*Numbers next to given node* percentage bootstrap support: *plain numbers* likelihood values out of 100 iterations; *underlined numbers* parsimony values out of 500 iterations; *italicised numbers* neighbor-joining values with 500 iterations (bootstrap values $<60\%$ are not shown); *values between OTUs* for *Ridgeia piscesea* and *Escarpia spicata* indicate bootstrap support for respective taxa; however, branch lengths in these situations are extremely short and, to some degree, values may result from strictly bifurcating assumptions made by "neighbor" and "fastDNAml boot" programs

Presently, the higher-order relationships between Annelida, Pogonophora and other protostome invertebrates remain ambiguous. The paraphyletic relationship between the pogonophoran taxa included in this study is not without precedent. Based on morphological criteria, Rouse and Fauchald (1995) discussed the possible paraphyly of the frenulate pogonophorans and the Polychaeta. Monophyly of the Annelida seems increasingly unsupported, as is evidenced by the present results. Nevertheless, a problem with the present analysis and all other molecular studies to date is a strong representational bias regarding the taxa included for phylogenetic analyses. The Annelida comprise $>14\,000$ species and the Pogonophora >140 species. The representation of these groups in molecular analyses has been extremely limited, leading perhaps to biases that may have profound effects on phylogenetic reconstructions of these diverse groups (see Swofford and Olsen 1990).

Unfortunately, the fossil record for soft-bodied worms is notoriously poor and incomplete (see Bengtson 1994 and other studies cited therein), and thus it is difficult to make reasonable guesses concerning divergence times for these taxa based on morphological grounds. However the molecular data suggest that vestimentiferans appear to constitute a recent group compared to the outgroup taxa (Fig. 1). Furthermore, the short internal and terminal branches within the vestimentiferan clade suggest that the present distribution of taxa can be accounted for by a recent rapid radiation. COI is one of the most conservative genes in the mitochondrial genome with respect to amino acid substitution. We found $<1\%$ sequence divergence among the extant vestimentiferans. According to the estimates of COI a.a. sequence divergence presented by Wray et al. (1996), one interpretation of our data is that the Vestimentifera as a taxon may be <100 million years old, indicating a more recent divergence than is traditionally recognized.

Table 2 Pairwise distance comparisons for nucleotide data (including third-position transitions) (*Above diagonal* genetic distances corrected with the Kimura two-parameter model; *below diagonal* absolute pairwise differences) OTU labels and full specific names in Table 1

	Rpa 9N	*Rpa* 18S	*Ela*	*Esp* seep	*Esp* vent	*Lba* MV	*Lba* OM	*Lco*	*Oal*	*Rpi* SJR	*Rpi* SER	*Rsp* SER	*Tje*	Nikko vest.	*Gbr*	Loihi pogo	*Hme*	*Ttu*	*Ppa*
Rpa 9N		0.000	0.194	0.198	0.196	0.183	0.186	0.187	0.174	0.175	0.173	0.173	0.153	0.189	0.259	0.231	0.481	0.401	0.390
Rpa 18S	0		0.194	0.198	0.196	0.184	0.188	0.190	0.175	0.179	0.174	0.175	0.153	0.189	0.265	0.228	0.480	0.402	0.390
Ela	107	109		0.003	0.002	0.160	0.162	0.157	0.158	0.195	0.188	0.190	0.146	0.153	0.247	0.205	0.492	0.390	0.386
Esp seep	109	111	2		0.002	0.160	0.162	0.157	0.158	0.195	0.188	0.189	0.150	0.153	0.245	0.201	0.491	0.384	0.390
Esp vent	108	110	1	1		0.158	0.160	0.155	0.156	0.193	0.186	0.187	0.148	0.151	0.245	0.203	0.488	0.387	0.387
Lba MV	100	102	91	91	90		0.003	0.050	0.167	0.167	0.163	0.163	0.165	0.048	0.238	0.203	0.487	0.386	0.356
Lba OM	102	104	92	92	91	2		0.050	0.171	0.171	0.167	0.167	0.171	0.049	0.242	0.209	0.498	0.388	0.357
Lco	103	106	90	90	89	31	31		0.162	0.166	0.161	0.161	0.163	0.053	0.238	0.204	0.501	0.373	0.360
Oal	96	97	89	89	88	93	95	91		0.137	0.131	0.131	0.164	0.175	0.260	0.219	0.486	0.385	0.401
Rpi SJR	97	99	108	108	107	93	95	93	80		0.005	0.005	0.155	0.174	0.253	0.220	0.499	0.408	0.404
Rpi SER	96	98	106	106	105	92	94	92	77	3		0.000	0.153	0.169	0.253	0.216	0.488	0.406	0.398
Rsp SER	96	99	107	107	106	92	94	92	77	3	0		0.153	0.169	0.253	0.215	0.486	0.407	0.397
Tje	86	87	84	86	85	93	96	93	92	88	88	88		0.165	0.252	0.208	0.494	0.380	0.357
Nikko vest.	103	105	87	87	86	30	30	33	97	96	95	95	93		0.222	0.204	0.470	0.368	0.341
Gbr	133	138	130	129	129	125	127	126	134	131	133	133	132	118		0.274	0.385	0.399	0.365
Loihi pogo.	123	124	113	111	112	111	114	113	118	118	118	118	113	112	141		0.489	0.430	0.386
Hme	207	211	214	214	213	209	212	215	208	213	213	213	213	203	176	213		0.463	0.433
Ttu	185	189	184	182	183	181	182	179	180	188	190	191	180	175	186	198	209		0.370
Ppa	179	183	182	184	183	170	171	174	184	183	184	184	170	165	173	184	198	180	

Alternatively, one can argue that the rate of evolutionary change of the COI gene within the Vestimentifera has slowed substantially. Because this estimate is based on a single gene sequence, the evolutionary interpretation must be viewed with caution. Currently, we are focusing on additional genetic markers to help resolve this issue of vestimentiferan age.

The present COI nucleotide-sequence data provides good resolution of relationships among vestimentiferan taxa (Fig. 2). The illustrated topology was fully supported by all three types of phylogenetic analyses (ML, NJ, and MP). Genera associated with eastern Pacific hydrothermal vents formed a monophyletic clade with two groups of sister taxa: *Oasisia* and *Ridgeia* and *Riftia* and *Tevnia*. The genera typically associated with cold-water sulfide/hydrocarbon seeps [*Lamellibrachia* (including the undescribed species from Nikko Seamount) and *Escarpia*] were paraphyletic and basal to the vent clade. Although the clustering of *Tevnia* with *Riftia* only received marginal bootstrap support, the monophyly of the *Oasisia–Ridgeia* clade is strongly supported by bootstrap values of 93, 84 and 99% for ML, MP and NJ analyses, respectively. The present relationships are different from the tree generated by Williams et al. (1993) based on 322 bp of the nuclear large-subunit (28S) rDNA. Their 28S rDNA data placed *Ridgeia* and *Tevnia* as sister taxa, with *Riftia* basal to the "vent" clade; however, they did not include *Oasisia* in their analysis. Furthermore, the assignment of higher taxonomic levels by Jones (1985) does not reflect genealogical relationships based on the COI sequences. His assignment of *Riftia pachyptila* to the monogeneric taxon Axonobrachia, based on arrangement of the branchial plume and its vascular system, is unsupported by the present data. In addition, our data do not support *Oasisia* and *Tevnia* as distinct from *Ridgeia*.

A major feature seen in the diversification of these species of vestimentiferan tubeworms was the association with specific kinds of deep-sea environments. Together, the genera *Riftia*, *Oasisia*, *Ridgeia*, and *Tevnia* form a clade that is endemic to hydrothermal vents of the eastern Pacific. In contrast, the genera *Escarpia* and *Lamellibrachia* are found in soft sediments associated with cold-water sulfide/hydrocarbon seeps. A recent molecular study (Feldman et al. 1997a) of bacterial endosymbionts associated with these vestimentiferans revealed a parallel, but not concurrent, subdivision of their bacteria (i.e. they did not coevolve). All species of vent-endemic tubeworms contain one endosymbiont species, while the vent-associated tubeworms contain another species which is markedly genetically different.

The two seep-associated genera are paraphyletic and basal to the vent-clade. However, some members of the "seep" genera appear to be opportunistic, also occurring in sedimented basins near hydrothermal vents. For example, *Escarpia spicata* is found near the Guaymas Basin hydrothermal vents in the Gulf of California, and *Lamellibrachia barhami* occurs near hydrothermal vents in Middle Valley on the Juan de Fuca Ridge (Black et al.

1997). Kojima et al. (1997) provide similar examples for *Escarpia* and *Lamellibrachia* in the vicinity of Japan. The best evidence for the opportunistic nature of seep-associated tubeworms was the recent discovery of *E. spicata* living in sulfide-rich sediments associated with a whale carcass (Feldman et al. 1997b). Perhaps the opportunistic nature of some seep-associated tubeworms may account for their invasion of hydrothermal areas. We suspect the present vent-endemic clade arose from such an opportunistic ancestor. This evolutionary pathway is also found in deep-sea mussels (Craddock et al. 1995). Seep-associated species are basal and paraphyletic, whereas hydrothermal vent-endemic species form a monophyletic and derived group. It will be interesting to see if this pattern of evolution is repeated in other groups such as vesicomyid clams and bresiliid shrimp.

Acknowledgements We thank the ship and submersible crews of the "Atlantis II/Alvin", "Nadir/Nautile", and the "Yokosuka/Shinkai 6500" for their hospitality and assistance. Our thanks to E. Southward who was fortuitously onboard during the collection of our sole *Galathealinum brachiosum* specimen, and to C. Moyer of the University of Hawaii for providing specimens. A. Trivedi and C. Di Meo assisted with PCR and sequencing. This is Contribution No. 97-09 of the Institute of Marine and Coastal Sciences, and New Jersey Agricultural Experiment Station Publication No. D-32104-5-97, supported by state funds and by NSF Grants OCE89-17311, OCE92-17026, and OCE93-02205.

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